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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

USB 96 AK IDM APC

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

09/194053

INTERNATIONAL APPLICATION NO.

PCT/EP97/02703

INTERNATIONAL FILING DATE

15 MAY 1997

PRIORITY DATE CLAIMED

21 MAY 1996

TITLE OF INVENTION ANTIGEN PRESENTING CELLS, A PROCESS FOR PREPARING THE SAME
AND THEIR USE AS CELLULAR VACCINES

APPLICANT(S) FOR DO/EO/US

Mohamed CHOKRI, Jacques BARTHOLEYNS and Jean-Loup ROMET-LEMONNE

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: International Preliminary Examination Report (PCT/IPEA/409)
International Search Report (PCT/ISA/210)
Patent Application Data Entry Format Sheet
2 Small Entity Forms

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)

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17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

Neither international preliminary examination fee (37 CFR 1.482)
nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO
and International Search Report not prepared by the EPO or JPO \$ 970.00

International preliminary examination fee (37 CFR 1.482) not paid to
USPTO but International Search Report prepared by the EPO or JPO \$840.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO
but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$760.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO
but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$670.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO
and all claims satisfied provisions of PCT Article 33(1)-(4) \$96.00

ENTER APPROPRIATE BASIC FEE AMOUNT =

CALCULATIONS PTO USE ONLY

\$ 840

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30
months from the earliest claimed priority date (37 CFR 1.492(e)).

\$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$
Total claims	21 - 20 =	1	x \$18.00	\$ 9
Independent claims	3 - 3 =	0	x \$78.00	\$
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00	\$
TOTAL OF ABOVE CALCULATIONS =				\$ 849
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				\$ 420
SUBTOTAL =				\$ 429
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$
TOTAL NATIONAL FEE =				\$ 429
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$ 40
TOTAL FEES ENCLOSED =				\$ 469
				Amount to be refunded: \$
				charged: \$

\$

\$

\$

\$

\$ 849

\$ 420

+

\$ 429

\$

\$ 429

\$ 40

\$ 469

\$

\$

a. ☒ A check in the amount of \$ 469 to cover the above fees is enclosed.b. ☐ Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees.
A duplicate copy of this sheet is enclosed.c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 25-0120. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

November 23, 1998

Young & Thompson
745 South 23rd Street
2nd Floor
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Benoit Castel
SIGNATURE

Benoit Castel
NAME

35,041
REGISTRATION NUMBER

PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Mohamed CHOKRI et al.

Serial No. (unknown)

Filed herewith

NEW ANTIGEN PRESENTING CELL, A
PROCESS FOR PREPARING THE SAME AND
THEIR USE AS CELLULAR VACCINES

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents

Washington, D.C. 20231

Sir:

Prior to calculation of the filing fee, please
amend these claims as follows:

IN THE CLAIMS:

Replace all of the claims now in the case with the
following new claims:

--23. MD-APCs which have the following proper-
ties:

- they present on their surface:

* antigen CD14 and CD64 with a mean intensity of about 5 to
about 200,

* antigen CD80 and CD86 with a mean intensity of about 20
to about 200,

* antigen CD40 and mannose receptor with a mean intensity
of 50 to 500,

- they are substantially devoid of the surface antigens CD1a and
CD1c, the presence and mean intensities respectively of CD14,

CD64, CD80, CD86 being for instance determined by immunofluorescence staining and flow cytometry analysis,

- they present a phagocytosis property such as determined by the following test: said phagocytosis capacity being evaluated by an uptake of formalin fixed yeast, for example by culturing macrophages for 2 hours, adding yeast in 1/10 macrophages/yeast ratio and incubating at 37°C, 5% CO₂ atmosphere for 2-3 hours fixing by the May-Grünwald-Giemsa (MGG) staining, and the percentage of phagocytic MD-APCs being quantified for instance by microscopic analysis,

- they have the property of stimulating the proliferation of allogenic lymphocytes such as determined by the following test: allogenic primary mixed lymphocytes reaction (MLR) was carried out in 96-well microtiter plates by adding different numbers (2×10^3 to 2×10^5 in 100 μ l medium/well) of MD-APCs to 2×10^5 in 100 μ l medium/well of allogenic T cells purified from buffy coats and after 5 days incubation at 37°C, cell proliferation was assessed by a colorimetric method, such as the hydrolysis of tetrazolium salt WST-1 (Boehringer Mannheim, Germany), (slightly red) to Formozan (dark red).

--24. MD-APCs according to claim 23, which present, on their surface, antigen MHC-II with a mean intensity of about 100 to about 400, such as determined by immunofluorescence staining and flow cytometry analysis.

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--25. MD-APCs according to claim 23, which are substantially devoid of surface antigen CD83, such as determined by immunofluorescence staining and flow cytometry analysis.

--26. MD-APCs according to claim 23, which present adherent properties such as determined by the following test:

the macrophages are cultured for 2 h in culture medium (I.M.D.M. or R.P.M.I.) on plastic flasks and the percentage (%) of adherent cells is quantified for instance by microscopic analysis.

--27. MD-APCs culture wherein:

- about 10% to about 50% of the MD-APCs present antigen CD14 on their surface,
- about 10% to about 50% of the MD-APCs present antigen CD46 on their surface,
- about 30% to about 100% of the MD-APCs present antigens CD80 and CD86 on their surface,
- about 80% to about 100% of the MD-APCs present antigen MHC-II on their surface,
- about 70% to about 100% of the MD-APCs present adherent properties,
- about 30% to about 100% of the MD-APCs present a phagocytosis property,

each MD-APCs having the above-mentioned properties being such that said properties are expressed according to the intensities as specified in claim 23.

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--28. Process for preparing a composition comprising MD-APCs according to claim 23, comprising the culture of mononuclear cells in a culture medium containing chemical ligands of mononuclear cells, such as histamine or histamine agonist and a H_2 antagonist, in combination or not with "additional" GM-CSF, or other chemical ligands interacting with mononuclear cells and allowing differentiation into MD-APCs, such as detoxified LPS such as lipid A, C3 and other ligands of complement receptors, taxols, oxydoreductors such as flavenoids or polyphenols, ligands to CD40, to the TNF receptors or to vitamin D3 receptors.

--29. Process according to claim 28, wherein the culture medium contains chemical ligands of mononuclear cells, for example histamine and cimetidine or a H_2 antagonist without "additional" GM-CSF, histamine being present at a concentration of about 10^{-2} M to about 10^{-6} M, preferably of 10^{-4} M, and cimetidine or the H_2 antagonist being present at a concentration of about 10^{-4} M to about 10^{-9} M, preferably about 10^{-6} M.

--30. Process according to claims 28, wherein the culture medium contains chemicals ligands of mononuclear cells, for example histamine and cimetidine or H_2 antagonist, in combination with "additional" GM-CSF, histamine being present at a concentration of about 10^{-2} M to about 10^{-6} M, preferably of about 10^{-4} M, cimetidine or the H_2 antagonist being present at a concentration of about 10^{-4} M to about 10^{-9} M, preferably of about 10^{-6}

M, and additional GM-CSF being present at a concentration of about 50 U/ml to about 2000 U/ml, preferably of about 500 U/ml.

--31. Process according to claim 28, comprising:

- isolation of leukocytes, from healthy donors or from patients, from peripheral blood apheresis and removal platelets and anticoagulants from the apheresis product,
- isolation of mononuclear cell (monocytes + lymphocytes) from red cells and granulocytes in order to have less than 10% granulocytes and less than 5% red cells,
- culture of the mononuclear cells obtained at the previous stage by placing them in an appropriate culture medium containing chemical ligands of mononuclear cells, such as histamine or an agonist of histamine, an H_2 antagonist, such as cimetidine, in combination or not with GM-CSF, for a time sufficient to obtain differentiated MD-APCs, preferably for about 5 to 15 days, and possibly separating the MD-APCs from the lymphocytes, and recovering the MD-APCs or the macrophages and lymphocytes.

--32. Process according to claim 28, wherein the culture medium of MD-APCs is added

- with crude antigens, for instance autologous tumor membrane, killed tumoral cells, bacterial capsides, viral homogenates cleared from nucleic acids,
- specific peptides against which an immune response is desired,
- cDNA or genetic material linked to vectors (for example gluconated polylysine) to allow transfection of the MD-APCs with

material coding for the relevant peptide or protein to be presented on the macrophage membrane and against which an immune response is desired,

- or bispecific antibodies targeting on the one side, a surface antigen of the MD-APCs and, on the other side, a relevant antigen against which an immune response is desired.

--33. MD-APCs liable to be obtained according to the process of claim 28.

--34. Pharmaceutical compositions containing as active substance, MD-APCs according to claim 23.

--35. Cellular vaccine compositions containing as active substance, MD-APCs according to claim 23.

--36. Medium containing elements necessary for the growth and differentiation of monocytes into MD-APCs according to claim 23, and in addition containing chemical ligands of mononuclear cells, for example histamine, cimetidine in combination or not with GM-CSF.

--37. Cell processor kit containing:

- means for the recovery of lymphocytes and monocytes free of contaminants,
- appropriate buffer and wash solutions and possibly appropriate means for the conservation of MD-APCs,
- means for preparing a culture for the monocytes and possibly the lymphocytes and containing chemical ligands of mononuclear

cells, for example histamine, cimetidine or a H₂ antagonist in combination or not with GM-CSF,

- possibly means for transfection of cultured cells and means for targeting antigens to MD-APCs.

--38. Cell processor or kit according to claim 37, containing:

- means for recovering and centrifuging blood to obtain a leukocyte concentrate,
- means for separating lymphocytes and monocytes from the other white cells and for eliminating the contaminating red cells,
- culture medium for MD-APCs and possibly lymphocytes with complements and particularly chemical ligands of mononuclear cells, for example histamine and cimetidine or a H₂ antagonist, in combination or not with GM-CSF,
- appropriate means for the conservation of MD-APCs,
- appropriate buffer and wash solution.

--39. Products containing MD-APCs according to claim 23, and lymphocytes, as a combined preparation for simultaneous, separate or sequential use in cell therapy.

--40. Products according to claim 39, characterized in that they contain the MD-APCs and the lymphocytes in a ratio of at least 20% to 50% of MD-APCs expressed in cell number.

--41. Method for the clinical treatment, comprising the administration of an appropriate amount of MD-APCs

according to claim 23, and preferably in an amount of about 10^8 to about 5×10^9 MD-APCs.

--42. Method according to claim 41 for the treatment of any disorder, comprising the administration of lymphocytes in an amount of about 4×10^9 to about 10×10^9 lymphocytes.

--43. Use of chemical ligands of mononuclear cells, for example an agonist of histamine, in particular histamine, and a H_2 antagonist, in particular cimetidine, in combination or not with GM-CSF, for the preparation of MD-APCs having the following properties:

- they present on their surface:
 - * antigen CD14 and CD64 with a mean intensity of about 5 to about 200,
 - * antigen CD80 and CD86 with a mean intensity of about 20 to about 200,
 - * antigen CD40 and mannose receptor with a mean intensity of 50 to 500,
- they are substantially devoid of the surface antigens CD1a and CD1c, the presence and mean intensities respectively of CD14, CD64, CD80, CD86 and the absence of CD1a and CD1c being for instance determined by immunofluorescence staining and flow cytometry analysis,
- they present high phagocytosis property such as determined by the following test: said phagocytosis capacity being evaluated

by an uptake of formalin fixed yeast, for example by culturing MD-APCs for 2 hours to select adherent cells, adding yeast in 1/10 mMD-APCs/yeast ratio and incubating at 37°C, 5% CO₂ atmosphere for 2-3 hours fixing by the May-Grünwald-Giemsa (MGG) staining and the percentage of phagocytic MD-APCs being quantified for instance by microscopic analysis,

- they have the property of stimulating the proliferation of allogenic lymphocytes such as determined by the following test: allogenic primary mixed lymphocytes reaction (MLR) was carried out in 96-well microtiter plates by adding different numbers (2×10^3 to 2×10^5 in 100 μ l medium/well) of MD-APCs to 2×10^5 in 100 μ l medium/well of allogenic T cells purified from buffy coats and after 5 days incubation 37°C, cell proliferation was assessed by a colorimetric method, such as the cleavage of tetrazolium salt WST-1 (slightly red) to Formozan (dark red) or such as Brdu incorporation during DNA synthesis.--

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R E M A R K S

The above changes in the claims merely place the national phase application in the same condition as it was during Chapter II of the international phase, with the multiple dependencies being removed.

Respectfully submitted,

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November 23, 1998

NEW ANTIGEN PRESENTING CELLS, A PROCESS FOR PREPARING
THE SAME AND THEIR USE AS CELLULAR VACCINES.

The invention relates to new antigen presenting cells, a process for preparing the same and their use as cellular vaccines.

Macrophages are recognized since their description by Metchnikoff (Immunity in Infective Diseases, Cambridge Press, 1905) as cells which very effectively phagocytose (interiorise) and digest exogenous particles (antigens, cell debris, bacteria). They also secrete a variety of immunoeffector monokines. They have therefore a central role in initiating the non-specific and the specific immune responses. The recovery of large quantities of human macrophages differentiated in culture from blood monocytes has already been described (International application N° PCT/EP93/01232, refs. 1-6). These macrophages express typical antigens and functions and have been fully characterized (refs. 7-14).

Macrophages activated in the presence of IFN γ (Macrophage Activated Killers : MAK) elicited selective cytostasis and cytotoxicity for a large number of human tumors even at low effector/target ratio. In murine models, murine activated macrophages given locally in the tumor or its vicinity infiltrated the tumor mass, inhibited tumor growth and decreased metastatic development. Human macrophages also inhibited the growth of human tumors engrafted in nude or SCID mice ; this effect was achieved after local or systemic injection of a low number of macrophages (less than 1 million MAK) for mice with macroscopic tumors (ref. 15-20).

Patients with metastatic cancer were infused systemically or intraperitoneally with 10^8 to 4×10^9 autologous MAK. Tumoricidal monocytes (AKM) were also infused intraperitoneally in patients with colorectal carcinomas. The clinical tolerance of MAK was excellent with minor side effects such as low grade fever and chills and no autoimmune nor acute phase reactivity. No complete antitumoral response was reported ; improved prognosis and prolonged disease free intervals were described after intraperitoneal injection of AKM, while tumor necrosis, stabilisation, reduction of ascitic fluid and change in chemioresistance were seen after MAK therapy (refs. 21 - 26).

The recognized limitation of these macrophages is that they are not very potent in the priming of a specific immune response against a specific exogenous antigen or tumor by stimulation of MHC class I restricted cytotoxic

T lymphocytes (CD8 +). They are more efficient in presenting antigens in the context of MHC class II molecules to T helper lymphocytes (CD4 +).

However these macrophages have the potential to process and present soluble antigens by the exogenous pathway of phagocytes but high concentrations of proteins or antigens are required (refs. 38 - 42, 45 - 47, 50).

Cells expressing these functions of phagocytosis, digestion, processing, and presentation at a high level would be required for the development of cellular vaccines.

Dendritic cells are characterized by their morphology (dendrites) and a few membrane antigens, and are considered as professional antigen - presenting cells resident in tissues. They are the most potent cells for the stimulation of primary T - lymphocyte immune responses (refs. 43 - 44, 47 - 49, + Dendritic cells in fundamental and clinical immunology. 1995, Plenum Press N.Y., Banchereau and Schmitt Editors).

Dendritic cell precursors arise from bone marrow and can be found in blood and lymph. Dendritic cells derived from these origins can be obtained by culture in the presence of GM - CSF + IL4 + TNF. They will then exhibit differences related to their maturation state and microenvironment.

The dendritic cells which can be derived from blood are most potent to induce allogenic mixed lymphocyte reactions and to stimulate naive T lymphocytes. However, these classical dendritic cells are technically relatively difficult to obtain and are poorly phagocytosing.

In contrast to macrophages, they do not express of CD14 and CD64 (high affinity F γ receptor).

To circumvent the problem of poor phagocytoses and processing of particular antigens by dendritic cells, these cells have been pulsed with small peptides fixed on MHC-I molecules to induce primary immune reaction and vaccination against new antigenic peptides (ref. 51).

Obtaining dendritic cells by *in vitro* differentiation requires the presence of GM-CSF and of a second cytokine that can be IL 4 (ref. 44) or IL 13 (ref. 49), plus TNF.

One of the aims of the invention is to provide cells with high phagocytosis, and efficient antigen presentation.

Another aim of the invention is to provide very potent antigen presenting cells derived from human blood monocytes.

Another aim of the invention is to provide cells presenting membrane receptors, allowing targeting and increased antigen presentation with the use of bispecific antibodies (refs. 27 - 31, 37).

Another aim of the invention is to provide cells which can be transfected with cDNA to be used in gene therapy (ref. 32 - 36).

Another aim of the invention is to provide a method allowing the recovery from human blood of cells of the macrophage lineage with high phagocytosis, digestive activity, processing MHC class I and class II antigen presentation (ref. 46, 50, 52).

Another aim of the invention is to provide a reproducible method allowing to obtain the above defined cells, said process not requiring combination of exogenous cytokines, defined media and recipients constituting a cell processor.

Another aim of the invention is to provide cellular vaccines demonstrating the high phagocytosis, processing, MHC-II peptide presentation of the macrophages and also the potent MHC-I T lymphocyte stimulation with high level of accessory molecules for presentation of the dendritic cells.

The invention relates to macrophages which have the following properties :

- they present on their surface :

* antigen CD14 with a mean intensity of about 20 to about 200,

* antigen CD64 with a mean intensity of about 20 to about 200,

- they are substantially devoid of the surface antigens CD1a and CD1c,

the presence and mean intensities respectively of CD14, CD64 and the absence of CD1a and CD1c being for instance determined by immunofluorescence staining and flow cytometry analysis,

- they present a phagocytosis property such as determined by the following test: said phagocytosis capacity being evaluated by an uptake of formalin fixed yeast, for example by culturing macrophages for 2 hours, adding yeast in 1/10 macrophage/yeast ratio and incubating at 37°C, 5% CO₂ atmosphere for 2-3 hours fixing by the May-Grünwald-Giemsa (MGG) staining, and the percentage of phagocytic macrophages being quantified for instance by microscopic analysis,

- they have the property of stimulating the proliferation of allogenic lymphocytes such as determined by the following test :

allogenic primary mixed lymphocytes reaction (MLR) was carried out in 96-well microtiter plates by adding increasing numbers (2×10^3 to 2×10^5) in 100 μ l medium/well of macrophages to 2×10^5 in 100 μ l medium/well of allogenic T cells purified from buffy coats and after 5 days incubation at 37°C, cell proliferation was assessed by a colorimetric method, such as the hydrolysis of tetrazolium salt WST-1 (Boehringer Mannheim, Germany), (slightly red) to Formozan (dark red).

More generally, the invention relates to monocytes derived antigen presenting cells (MD-APCs), particularly macrophages which have the following properties :

- they present on their surface :

5 * antigens CD14 and CD64 with a mean intensity of about 5 to about 200,
 * antigen CD80 and CD86 with a mean intensity of about 20 to about 200,

 * antigen CD40 and mannose receptor with a mean intensity of 50 to 500,
- they are substantially devoid of the surface antigens CD1a and CD1c,

10 the presence and mean intensities respectively of CD14, CD64, CD80 and CD86, and the absence of CD1a and CD1c being for instance determined by immunofluorescence staining and flow cytometry analysis,

- they present a phagocytosis property such as determined by the following test: said phagocytosis capacity being evaluated by an uptake of formalin fixed yeast,
15 for example by culturing MD-APCs for 2 hours, adding yeast in 1/10 MD-APCs/yeast ratio and incubating at 37°C, 5% CO₂ atmosphere for 2-3 hours fixing by the May-Grünwald-Giemsa (MGG) staining, and the percentage of phagocytic MD-APCs being quantified for instance by microscopic analysis,

20 - they have the property of stimulating the proliferation of allogenic lymphocytes such as determined by the following test :

allogenic primary mixed lymphocytes reaction (MLR) was carried out in 96-well microtiter plates by adding increasing numbers (2×10^3 to 2×10^5) in 100 μ l medium/well of MD-APCs to 2×10^5 in 100 μ l medium/well of allogenic
25 T cells purified from buffy coats and after 5 days incubation at 37°C, cell proliferation was assessed by measurement of Brdu incorporation during DNA synthesis by ELISA method (Boehringer Mannheim, Germany).

In the context of the present invention, the expression "macrophages" designates not only macrophages but antigen presenting cells which derive from monocytes and which will be hereafter designated by MD-APC.
30

The expression "substantially devoid of surface antigens CD1a and CD1c" means either that there is no such surface antigens or that there is only a dim intensity for these surface antigens, with said dim intensity corresponding to about 10 times less than the intensity obtained in the presence of such surface
35 antigens, as determined in immunofluorescence analysis, or with said intensity being lower than 20.

In the following of the text, it will be often referred to "the absence of surface antigen CD1a and CD1c", which is to be understood as "substantially devoid of such antigens" as explained above (intensity lower than 20).

The invention also relates to MD-APCs which present, on their surface, antigen MHC-II with a mean intensity of about 100 to about 600, such as determined by immunofluorescence staining and flow cytometry analysis.

The invention also relates to macrophages which are substantially devoid of surface antigen CD83, such as determined by immunofluorescence staining and flow cytometry analysis.

The expression "substantially devoid of surface antigen CD83" corresponds to an intensity lower than 20.

The MD-APCs of the invention present adherent properties such as determined by the following test :

the MD-APCs are cultured for 2 h in culture medium (I.M.D.M. or R.P.M.I.) on plastic flasks and the percentage (%) of adherent cells is quantified for instance by microscopic analysis.

The culture media I.M.D.M. and R.P.M.I. are commercially available.

The invention relates to a MD-APCs culture wherein :

- about 10% to about 50% of the MD-APCs present antigen CD14 on their surface,
- about 10% to about 50% of the MD-APCs present antigen CD64 on their surface,
- about 80% to about 100% of the MD-APCs present antigen MHC-II on their surface,
- about 70% to about 100% of the MD-APCs present adherent properties,
- about 30% to about 100% of the MD-APCs present antigens CD80 and CD86 on their surface,
- about 30% to about 100% of the MD-APCs present high phagocytosis property,

each macrophage having the above-mentioned properties being such that said properties are expressed according to the intensities as specified above.

The invention also relates to a process for preparing a composition of macrophages which comprises the culture of mononuclear cells in a culture medium containing histamine or histamine agonist (H_1 in action) and a H_2 antagonist, in combination or not with "additional" GM-CSF.

The invention also relates to a process for preparing a composition of MD-APCs which comprises the culture of mononuclear cells in a culture medium containing a chemical ligand having receptors on the membrane of

mononuclear cells, for example histamine or histamine agonist (H_1 in action) and a H_2 antagonist, in combination or not with "additional" GM-CSF.

An example of histamine agonist is 2 methyl-histamine.

The expression "additional" corresponds to the fact that there is no GM-CSF added to the culture in standard condition, but this does not exclude the fact that exogenous GM-CSF could be added in the culture to increase yield and functions of MD-APCs obtained.

As example of H_2 antagonist, one may cite cimetidine but also tiotidine, burimamide, metiamide, ranitidine.

As example of other chemical ligands interacting with mononuclear cells and allowing differentiation into MD-APCs, one may cite detoxified LPS such as lipid A, C3 and other ligands of complement receptors, taxols, oxydoreductors such as flavenoids or polyphenols, ligands to CD40, to the TNF receptors or to vitamin D3 receptors.

The invention also relates to a process wherein the culture medium contains chemical ligands, such as histamine and cimetidine or a H_2 antagonist without "additional" GM-CSF, histamine being present at a concentration of about 10^{-2} M to about 10^{-6} M, preferably of about 10^{-4} M, and cimetidine or the H_2 antagonist being present at a concentration of about 10^{-4} M to about 10^{-9} M, preferably of about 10^{-6} M.

When the concentration of histamine or cimetidine or other chemical ligands for membrane receptors is lower than the lower value of the given range, there is substantially no effect, i.e. less than 10% difference with cells cultured in the absence of histamine and cimetidine.

When the concentration of histamine or cimetidine is higher than the higher value of the given range, the culture medium becomes toxic.

When no additional GM-CSF is incorporated into the culture medium, GM-CSF secreted *in situ* by the MD-APCs can be present at a concentration of about 5 to about 500 U/ml.

The invention also relates to a process wherein the culture medium contains a chemical ligand, for example histamine and cimetidine or a H_2 antagonist, in combination with "additional" GM-CSF, histamine being present at a concentration of about 10^{-2} M to about 10^{-6} M, preferably of about 10^{-4} M, cimetidine or the H_2 antagonist being present at a concentration of about 10^{-4} M to about 10^{-9} M, preferably of about 10^{-6} M, and additional GM-CSF being present at a concentration of about 50 U/ml to about 1000 U/ml, preferably of about 500 U/ml.

When additional GM-CSF is incorporated into the culture, then the total concentration of GM-CSF is from about 50 U/ml to about 2000 U/ml, and preferably from about 50 U/ml to about 500 U/ml.

According to a particular embodiment of the invention, the culture medium does not comprise the following elements : exogenous cytokines such as IL4, IL10, TNF.

The invention also relates to a process comprising :

- isolation of leukocytes, from healthy donors or from patients, from peripheral blood by apheresis and removal of platelets and anticoagulant from the apheresis product,
- isolation of mononuclear cells (monocytes + lymphocytes) from red cells and granulocytes in order to have less than 10% granulocytes and less than 5% red cells,
- culture of the mononuclear cells obtained at the previous stage by placing them in an appropriate culture medium containing a chemical ligand of mononuclear cells, such as histamine or an agonist of histamine, an H₂ antagonist, such as cimetidine, in combination or not with GM-CSF, for a time sufficient to obtain differentiated MD-APCs, preferably for about 5 to 15 days, and possibly separating the MD-APCs from the lymphocytes, and recovering the MD-APCs or the MD-APCs and lymphocytes.

The invention also relates to a process wherein the culture medium of MD-APCs is added

- with crude antigens, for instance autologous tumor membrane, killed tumoral cells, bacterial capsides, viral homogenates cleared from nucleic acids,
- specific peptides against which an immune response is desired,
- cDNA or genetic material linked to vectors (for example gluconated polylysine) to allow transfection of the macrophage with material coding for the relevant peptide or protein to be presented on the MD-APCs membrane and against which an immune response is desired,
- or bispecific antibodies targeting on the one side, a surface antigen or a surface receptor of the MD-APCs and, on the other side, a relevant antigen against which an immune response is desired.

The invention also relates to MD-APCs liable to be obtained according to the process of the invention described hereabove.

The invention also relates to pharmaceutical compositions containing as active substance, MD-APCs according to the invention.

The invention also relates to cellular vaccine compositions containing as active substance, MD-APCs according to the invention.

The invention also relates to the medium containing elements necessary for the growth and differentiation of monocytes into MD-APCs according to the invention, and in addition containing chemical ligands of mononuclear cells, such as histamine, cimetidine in combination or not with GM-CSF.

As example of other chemical ligands interacting with mononuclear cells and allowing differentiation into MD-APCs, one may cite detoxified LPS such as lipid A, C3 and other ligands of complement receptors, taxols, oxydoreductors such as flavenoids or polyphenols, ligands to CD40, to the TNF receptors or to vitamin D3 receptors.

The invention also relates to a cell processor or a kit containing :

- means for the recovery of lymphocytes and monocytes free of contaminants,
- appropriate buffer and wash solutions and possibly appropriate means for the conservation of macrophages,
- means for preparing a culture for the monocytes and possibly the lymphocytes and containing chemical ligands of mononuclear cells, for example histamine, cimetidine or a H₂ antagonist in combination or not with GM-CSF,
- possibly means for transfection of cultured cells and means for targeting antigens to MD-APCs.

Regarding the conservation of MD-APCs, it can include freezing means for example in 10% glycerol or D.M.S.O. (dimethyl sulfoxyde) in the presence of autologous or AB⁺ serum.

The invention also relates to a cell processor or a kit as described above which contains :

- means for recovering and centrifuging blood to obtain a leukocyte concentrate,
- means for separating lymphocytes and monocytes from the other white cells and for eliminating the contaminating red cells,
- culture medium for MD-APCs and possibly lymphocytes with complements and particularly chemical ligands of mononuclear cells, such as histamine and cimetidine or a H₂ antagonist, in combination or not with GM-CSF,
- appropriate means for the conservation of macrophages,
- appropriate buffer and wash solution.

The invention also relates to products containing MD-APCs according to the invention, and lymphocytes, as a combined preparation for simultaneous, separate or sequential use in cell therapy.

The invention also relates to products as described hereabove which contain the MD-APCs and the lymphocytes in a ratio of at least 20% to 50% of MD-APCs expressed in cell number.

The invention also relates to bispecific antibodies liable to recognize an antigen of a MD-APCs of the invention and an antigen of a tumoral cell or of a pathogen which is to be targetted to said MD-APCs.

The invention also relates to a method for the clinical treatment, comprising the administration of an appropriate amount of MD-APCs according to the invention, and preferably in an amount of about 10^8 to about 5×10^9 MD-APCs.

The invention also relates to a method for the treatment of any disorder, comprising the administration of lymphocytes from the culture in an amount of about 4×10^9 to about 10×10^9 lymphocytes.

The invention also relates to the use of a chemical ligands of mononuclear cells, such as an agonist of histamine receptor, in particular histamine, and a H_2 antagonist, in particular cimetidine, in combination or not with GM-CSF, for the preparation of MD-APCs having the following properties :

- they present on their surface :

* antigens CD14 and CD64 with a mean intensity of about 5 to about 200,

* antigen CD80 and CD86 with a mean intensity of about 20 to about 200,

* antigen CD40 and mannose receptor with a mean intensity of 50 to 500,

- they are substantially devoid of the surface antigens CD1a and CD1c,

the presence and mean intensities respectively of CD14, CD64, CD80, CD86 and the absence of CD1a and CD1c being for instance determined by immunofluorescence staining and flow cytometry analysis,

- they present high phagocytosis property such as determined by the following test:

said phagocytosis capacity being evaluated by an uptake of formalin fixed yeast, for example by culturing MD-APCs for 2 hours to select adherent cells, adding yeast in 1/10 macrophages to yeast ratio and incubating at 37°C , 5% CO_2 atmosphere for 2-3 hours fixing by the May-Grünwald-Giemsa (MGG) staining, and the percentage of phagocytic MD-APCs being quantified for instance by microscopic analysis,

- they have the property of stimulating the proliferation of allogenic lymphocytes such as determined by the following test :

allogenic primary mixed lymphocytes reaction (MLR) was carried out in 96-well microtiter plates by adding different numbers 2×10^3 to 2×10^5 in $100 \mu\text{l}$ medium/well of MD-APCs to 2×10^5 in $100 \mu\text{l}$ medium/well of allogenic T cells purified from buffy coats and after 5 days incubation at 37°C , cell proliferation

was assessed by a colorimetric method, such as the cleavage of tetrazolium salt WST-1 (slightly red) to Formozan (dark red).

The monocytes derived antigens presenting cells of the invention and the MD-APCs can be obtained as follows :

5 a) Isolation of leukocytes from blood by apheresis and elimination of platelets, by centrifugation. If cells collected have < 10% granulocytes and haematocrit < 5%, they can be seeded as such in culture. Otherwise mononuclear cells have to be prepared first by centrifugation on Ficoll Paque of density 1,077.

10 b) Mononuclear cells are then cultured for 5 to 15 days in hydrophobic bags ethylene vinyl acetate (E.V.A., Stedim) or polypropylene (Life Cell, Baxter), at 37°C, 5% CO₂. Cells are seeded at 5.10⁶/ml in I.M.D.M. (as previous) or equivalent medium supplemented with indomethacin (5x10⁻⁶M) mercaptoethanol (3x10⁻⁵M), non essential amino-acids (1%, Gibco) and 2 to 15 5% of reconstituted autologous or AB⁺ serum,

. with GM-CSF, 500 U/ml chemical ligands interacting with mononuclear cells and allowing differentiation into MD-APCs, such as detoxified LPS such as lipid A, C3 and other ligands of complement receptors, taxols, oxydoreductors such as flavenoids or polyphenols, ligands to CD40, to the 20 TNF receptors or to vitamin D3 receptors ; as example of ligands, histamine (10⁻⁴ M), cimetidine (10⁻⁶ M) or another H₂ antagonist of histamine, or with histamine, cimetidine in the absence of any exogenous cytokines. Endogenous cytokines are released by mononuclear cells stimulated by ligands.

. and exogenous antigens, peptides or transfectants (cDNA + vector) 25 coding for the relevant antigen.

c) After culture, the specific monocyte derived antigen presenting cells (MD-APCs) are centrifuged, washed and resuspended for injection in the patient, to induce humoral and cellular immune response against the antigen.

The specificity of the cellular vaccine is achieved during the *in vitro* 30 culture according to one of the following items.

a) culture of MD-APCs as explained above in b), in the presence of crude antigens, for example autologous tumor membrane, bacterial capsides, viral homogenates cleared from nucleic acids ;

b) culture of MD-APCs as explained above in b), in the presence of 35 specific peptides against which immune response would be beneficial,

c) or culture of MD-APCs as explained above in b), in the presence of cDNA or genetic material linked to vectors (for example gluconated

polyphosphine) to allow transfection of MD-APCs with material coding for the relevant peptide or protein to be presented on the membrane.

In order to obtain specific cellular vaccine, it is also possible at the end of the differentiation stage of the macrophage culture to add bispecific antibodies targeting a membrane antigen, or a surface receptor of MD-APCs on one side and the relevant antigen on the other side.

According to a preferred embodiment, the process of the invention comprises the following steps:

- isolation of leukocytes from blood of healthy subjects or patients by apheresis, to obtain the apheresis products (i.e. concentrated leukocytes),
- platelet elimination, for instance by centrifugation of the apheresis products, to obtain a leukocyte enriched product,
- separation, in the leukocyte enriched products, of the mononuclear cells on one hand, and of the contaminating red blood cells and granulocytes on the other hand,
- culture of the mononuclear cells (monocytes + lymphocytes) in a medium containing chemical ligands of mononuclear cells, such as histamine and cimetidine and GM-CSF for about 5 to 15 days, to obtain differentiated monocyte derived antigen presenting cells (MD-APCs).

The lymphocytes can be separated from the monocytes before the culture step.

The lymphocytes can be separated from the MD-APCs after the culture.

In the process of the invention, chemical ligands for mononuclear cells, such as histamine are used at a concentration of 10^{-2} M to about 10^{-6} M, preferably of about 10^{-4} M.

In the process of the invention, GM-CSF is used at a concentration of about 50 to about 1000 U/ml, particularly of about 100 to about 500 U/ml.

In the process of the invention, the culture medium is RPMI, IMDM, MEM, or DMEM selected for very low endotoxin content.

These media are commercially available.

Advantageously, the culture medium contains indomethacin (or another cyclo-oxygenase inhibitor) or/and cimetidine (an histamine H_2 antagonist) and/or at other chemical ligands of mononuclear cells.

An advantageous process for preparing the MD-APCs of the invention is the following:

Apheresis

Leukocytes from healthy subjects or from patients are isolated from peripheral blood by apheresis using the Cobe Spectra continuous-flow blood cell

separators keeping granulocytes contamination very low (< 10% and less than 5% red cells). The apheresis product is centrifuged for 10 min at 280 g in order to reduce platelet contamination. The platelet-enriched plasma is removed and leukocyte pellet resuspended in a phosphate buffer solution (PBS) containing

5 0.1% glucose, 0.17% PO_3HNa_2 , $2\text{H}_2\text{O}$, 0.27% $\text{PO}_3\text{H}_2\text{Na}$, 0.14% NH_4Cl , 0.78% NaCl (solution TS745 laboratoire Bruneau, France).

The enriched leukocyte pellet is obtained with an average of 7 to 1.5×10^{10} leukocytes (50% of mononuclear cells).

Isolation of mononuclear cells

10 If the collected leukocytes have more than 10% granulocytes contamination and/or 5% hematocrite, human mononuclear cells are separated from red blood cells and from contaminating granulocytes, by 15 min centrifugation at 1000 g on a COBE 2991 or Stericell cell processor using Ficoll Paque of density 1.077 (Pharmacia). After 3 washings in phosphate buffered

15 saline solution without calcium and magnesium, the monocytes are obtained with about 20% to 50% purity as shown by channelyser analysis (Coulter Margency-France).

Culture

Differentiated human MD-APC are obtained by 5-15 days in culture of

20 mononuclear cells in hydrophobic bags in E.V.A. (Ethylene Vinyl Acetate, STEDIM, Aubagne) or polypropylene (life cell-Baxter) at 37°C and 5% CO_2 , 95% humidified atmosphere. Total mononuclear cells are seeded at 5×10^6 cells/ml in Iscove modified medium (*I.M.D.M.*, Gibco) or equivalent medium supplemented by penicillin (100 UI/ml), streptomycine (100 $\mu\text{g}/\text{ml}$),

25 L-glutamine (2 mM, Gibco), pyruvic acid (2 mM, Gibco), Indomethacin (5×10^{-6} M, Sigma), cimetidine (10^{-8} to 10^{-4} M), histamine (10^{-6} to 10^{-2} M or other chemical ligands), mercaptoethanol (3×10^{-5} M, Gibco) non-essential amino-acids (1%, Gibco) and 2-5% of autologous or AB serum. The addition of GM-CSF (500 U/ml, SANDOZ) was done in comparative experiment.

30 According to a preferred embodiment, the process of the invention is such that killed tumoral cells are added into the culture medium simultaneously with monocytes, both cells coming preferably from the same patient, preferably at the ratio of about 1 million of killed tumoral cells/ml, with said killed tumoral cells being processed at the same time as macrophages.

35 The killed tumoral cells can then be processed simultaneously with the leukocytes, in an amount of about $1 \times 10^6/\text{ml}$.

This process allows to obtain MD-APCs and lymphocytes specific for the tumor, inducing very efficiently *in vivo* an immune response to these specific tumor cells.

The invention also relates to MD-APCs liable to be obtained according to the above-defined process.

The invention also relates to pharmaceutical compositions containing, as active substance, MD-APCs as defined above.

The invention also relates to a medium containing elements necessary for the growth and differentiation of monocytes into MD-APCs of the invention, and in addition chemical ligands for mononuclear cells, for example histamine and GM-CSF.

The monocyte derived antigens presenting cells of the invention can be part of a cell processor or a kit containing:

- means for the recovery of lymphocytes and monocytes free of contaminants;
- appropriate buffer and wash solutions, and possibly appropriate means for the conservation of MD-APCs ;
- means for preparing a culture medium for the monocytes and possibly the lymphocytes and containing chemical ligands for mononuclear cells, for example histamine and/or GM-CSF.

According to an advantageous embodiment of the invention, the cell processor or kit contains :

- means for recovering and centrifuging blood to obtain a leukocyte concentrate;
- means for separating lymphocytes and monocytes from the other white cells and for eliminating the contaminating red cells;
- culture medium for MD-APCs and possibly lymphocytes with complements and particularly and/or GM-CSF and possibly indomethacin and/or cimetidine ;
- appropriate means for the conservation of the MD-APCs ;
- appropriate buffer and wash solutions ;

The invention also relates to products containing MD-APCs according to the invention, and lymphocytes, as a combined preparation for simultaneous, separate or sequential use in adoptive immunotherapy.

According to an advantageous embodiment, the products of the invention as defined above are characterised in that they contain the MD-APCs and the lymphocytes in a ratio of at least 20% to 50% of MD-APCs expressed in cell number.

In this embodiment, the MD-APCs and the lymphocytes are both injected to a patient.

The invention also relates to bispecific antibodies liable to recognize an antigen of a MD-APC of the invention, for example FC γ RI (CD 64) and an antigen of a relevant antigen.

The bispecific antibodies can be prepared as described in Chokri et al. Res. Immunol. 143 (1992).

The bispecific antibodies can be injected at the same time as the MD-APCs of the invention, or can be pre-incubated with MD-APCs before injection.

The invention also relates to a method for the treatment of cancer and pathogens comprising the administration of an appropriate amount of MD-APCs according to the invention, and preferably in an amount of about 1×10^8 to about 5×10^9 MD-APCs.

Description of the Figure :

Figure 1a represents the allogenic T cell proliferation induced by MD-APCs of the invention recovered in the presence of histamine (10^{-4} M) and cimetidine (10^{-6} M) as adjuvant, with or without GM-CSF (500 U/ml) comparing to standard macrophages produced only in the presence of the GM-CSF (500 U/ml).

Figure 1b represents the stimulation by MD-APCs recovered in the presence of GM-CSF or GM-CSF + IL-13.

In Figure 1a, the optical density (450-690 nm) has been plotted against the ratio between the MD-APCs of the invention and the responder lymphocyte cells.

The clear dotted bars correspond to the presence of GM-CSF at 500 U/ml; the lighter grey bars correspond to the presence of histamine (10^{-4} M) and cimetidine (10^{-6} M).

The darker grey bars correspond to the presence of GM-CSF (500 U/ml), in combination with histamine (10^{-4} M) and cimetidine (10^{-6} M).

The results show that the capacity of the MD-APCs to stimulate the proliferation of allogenic lymphocytes is strongly increased. The stimulation of allogenic proliferation of lymphocytes by MD-APCs is very potent (at least 200% increase with respect to standard macrophages). The combination of histamine/cimetidine effect or IL-13 effect with GM-CSF can potentiate this activity (maximum of 300% increase with respect to the induction by macrophages).

In Figure 1b, the optical density (450-690 nm) has been plotted against the ratio between the MD-APCs of the invention and the responder lymphocyte cells. The curve with dark circles corresponds to the presence of GM-CSF/IL-13 and the curve with open circles correspond to the presence of GM-CSF.

EXAMPLE : Preparation of MD-APCs according to the invention :

1- Apheresis:

Leukocytes from healthy donors or from patients are isolated from peripheral blood by apheresis using COBE spectra blood cell separator keeping granulocytes contamination the lowest possible. The apheresis product is diluted in a phosphate buffered solution (PBS) (final volume = 450 ml). The apheresis product is centrifuged for 10 min at 280 g to remove platelets and anticoagulant.

2- Isolation of mononuclear cells:

If the collected cells have > 10% granulocytes contamination and/or > 5% haematocrit, they should be centrifuged over Ficoll (density = 1.077) to remove red cells and granulocytes.

3- Culture

The monocyte derived antigen presenting cells (MD-APCs) are obtained after 5 to 15 days in culture of mononuclear cells in hydrophobic bags in EVA (Ethylene Vinyl Acetate /STEDIM) or equivalent bags (Teflon) at 37°C and 5% CO₂ in humidified atmosphere. The mononuclear cells are seeded at 5x10⁶ cells/ml in Iscove modified medium (I.M.D.M. - Gibco) or equivalent supplemented by (see PCT/EP93 page 7) the addition of ligands for mononuclear cells, such as histamine (10⁻⁴ M) or analogues and cimetidine (10⁻⁶ M) or similar H₂ antagonist, in the presence or not of GM-CSF (500 U/ml).

As example of other chemical ligands interacting with mononuclear cells and allowing differentiation into MD-APCs, one may cite detoxified LPS such as lipid A, C3 and other ligands of complement receptors, taxols, oxydoreductors such as flavenoids or polyphenols, ligands to CD40, to the TNF receptors or to vitamin D3 receptors.

4- Characterization and functionality

a- Flow cytometry

After the maturation of MD-APCs, the phenotype analysis of the obtained cells was performed by flow cytometry using murine FITC or P.E labelled monoclonal antibodies directed against membrane proteins.

b- Mixed lymphocytes reactions (MLR)

To evaluate the induction of T cell response to MD-APCs, allogenic primary MLR was carried out in 96-Well microtiter plates by adding different numbers of MD-APCs to 2×10^5 allogenic T cells purified from buffy coats. After 5 days at 37°C , cell proliferation was assessed by a colorimetric methods such as WST-1.

c- Phagocytosis

The phagocytic capacity of the different cells obtained was evaluated by uptake of formalin fixed yeast. Briefly the cells were cultured for 2 hours to select adherent cells. The yeast was added at 1/10 macrophage/yeast ratio and incubated in 37°C , 5% CO_2 atmosphere for 2-3 h and then fixed by the May-Grünwald-Giemsa (MGG) staining. The percentage of phagocytic cells was quantified by microscopic analysis.

The results are gathered in table 1, table 2 and table 3.

Table 1
Yield (% of cells) differentiated in culture

Time of culture	(a) Hist/Cim	(b) Hist.Cim/GM
Day 4	71%	78%
Day 7	49%	48%
Day 11	19%	31%

Table 1 shows that there is a recovery of a large quantity of MD-APCs after 5 to 11 days of culture of mononuclear cells in hydrophobic bags, in the presence of histamine (10^{-4} M) and cimetidine (10^{-6} M) (a) and additional GM-CSF (500 U/ml) (b) as adjuvant. In comparison with production of mature macrophages in standard conditions, the recovery of MD-APC cells is in the same order.

Table 2
Phenotype of MD-APCs
produced under various conditions after 6 days of culture

Surface Ag	(a) Hist/Cim		(b) Hist. Cim/GM-CSF	
	%	MIF	%	MIF
CD1a	N		N	
CD1c	N		N	
CD83	N		N	
CD14	95	114	95	92
CD 54	96	40	96	40
CD 58	97	41	97	31
CD 64	43	35	91	46
HLA-DR	92	350	96	400

MIF = Mean Intensity Fluorescence.

% = percentage of positive cells.

N = Negative or weak signal.

This table corresponds to the immunofluorescence profile analysis by flow cytometry of MD-APCs generated in culture (6 days) under different conditions. The cells obtained under histamine (10^{-4} M) and cimetidine (10^{-6} M) conditions (a) are positive for macrophage markers (CD14, CD64 and HLA-DR). The combination with GM-CSF (b) does not change the phenotypic profile of the MD-APCs. They also clearly express CD54 and CD58. The CD80 (B7.1) and CD86 (B7.2) are also expressed on their membrane but in lower level. In contrast, CD1a, CD1c and CD83 which are positive markers of dendritic cells, are weakly expressed by the MD-APCs.

From these data, it can be confirmed that the antigen presenting cells generated in the culture system of the invention are different from dendritic cells.

Table 3

% of phagocytic cells after 3 h contact with fixed yeast

	Number of intracellular Yeast	(a) Hist/cim	(b) Hist.Cim/GM-CSF
5			
10	0	42 %	20%
	1-5	43.6%	43%
	6-10	11 %	27.5%
15	> 10	3.4%	9.5%

The MD-APCs are tested for the phagocytic activity using formalin fixed yeast. After 3 h incubation and MGG (May Grünwald Giemsa) staining, the intracellular particles are quantified by microscopic analysis. The MD-APCs generated in the presence of histamine/cimetidine (a) present an important capacity of phagocytosis (60%) and this capacity is increased in the presence of GM-CSF in the culture (80%) (b).

From the above-mentioned results, it is shown that human mononuclear cells cultured in the presence of histamine and cimetidine, in combination or not with GM-CSF mainly for one week, differentiate into mature antigen presenting cells. During the culture, these cells acquire a high level of accessory functions like.

The results of the invention indicate that the MD-APCs obtained in the culture system of the invention are different from the dendritic cells (DC), since DC have been described as FC (CD64) receptor negative, poorly adherent and non-phagocytic cells, possessing only a small number of lysosomes.

In contrast the MD-APCs of the invention :

- (a) show a high adherence capacity,
- (b) show an important phagocytic and processing activity,
- (c) express high level HLA-DR membrane antigens and a low level of CD1a and CD1c, which is strongly expressed by dendritic cells,

(d) are also positive for CD54, CD58, CD80 and CD86 membrane antigens,

(e) stimulate T-cell proliferation in allogenic MLR reaction, and this is in a far better way than macrophages obtained according to techniques of the prior art.

Table 4 hereafter gathers the comparative characterization of MD-APC of the invention on the one hand, and of dendritic cells on the other hand.

Table 4
Comparative characterization of Antigen Presenting Cells:

	Dendritic Cells		MD-APC	
			% cells	Intensity
Phenotype				
CD1a	+	-	0	
CD1c	+	-	0	
CD 83	+	-	0	
CD14	+/-	+	10 to 100	5 to 200
CD64	-	+	10 to 100	5 to 200
CMH-II	+++	+++	80 to 100	100 to 400
Functions:				
Adherence	-	++	70 to 90 %	
Phagocytosis	-	++	30 to 80 %	
Stimulation of MLR	+++	++		

Table 5

It gathers a complete phenotypic characterization of MD-APCs recovered after 6 days of culture according to the invention (analysis by flow cytometry).

Phenotype	% Cells	Mean fluo intensity
CD45	97,6	172
CD14	6,8	
CD3	13	
CD19	15	
CD56	3,8	
CD4-PE	95	
CD25	1,7	
CD45RO	99	
CD16	1,8	31
CD32	63	163
CD64	4	12
CD1a	31	216
CD1c	58	505
CD83	9	18
HLA-DR	99	266
HLA-I	99,6	582
CD40	98	991
CD80	78	64
CD86	99	744
IgG1-FITC	6,7	11
IgG1-PE	20	(16)55
IgG1-Cy5	19	(29)50
IgG2a-FITC	5,3	19
IgG1 i	1,6	75
IgG2a i	3,8	21
IgG2b i	3,2	15

AA/31

CLAIMS

1. MD-APCs which have the following properties :

- they present on their surface :

* antigen CD14 and CD64 with a mean intensity of about 5 to about 200,

* antigen CD80 and CD86 with a mean intensity of about 20 to about 200,

* antigen CD40 and mannose receptor with a mean intensity of 50 to 500,

- they are substantially devoid of the surface antigens CD1a and CD1c,

the presence and mean intensities respectively of CD14, CD64, CD80, CD86 being for instance determined by immunofluorescence staining and flow cytometry analysis,

- they present a phagocytosis property such as determined by the following test: said phagocytosis capacity being evaluated by an uptake of formalin fixed yeast, for example by culturing macrophages for 2 hours, adding yeast in 1/10 macrophages/yeast ratio and incubating at 37°C, 5% CO₂ atmosphere for 2-3 hours fixing by the May-Grünwald-Giemsa (MGG) staining, and the percentage of phagocytic MD-APCs being quantified for instance by microscopic analysis,

- they have the property of stimulating the proliferation of allogenic lymphocytes such as determined by the following test :

allogenic primary mixed lymphocytes reaction (MLR) was carried out in 96-well microtiter plates by adding different numbers (2×10^3 to 2×10^5 in 100 μ l medium/well) of MD-APCs to 2×10^5 in 100 μ l medium/well of allogenic T cells purified from buffy coats and after 5 days incubation at 37°C, cell proliferation was assessed by a colorimetric method, such as the hydrolysis of tetrazolium salt WST-1 (Boehringer Mannheim, Germany), (slightly red) to Formozan (dark red).

2. MD-APCs according to claim 1, which present, on their surface, antigen MHC-II with a mean intensity of about 100 to about 400, such as determined by immunofluorescence staining and flow cytometry analysis.

3. MD-APCs according to any one of claims 1 or 2, which are substantially devoid of surface antigen CD83, such as determined by immunofluorescence staining and flow cytometry analysis.

AMENDED SHEET

4. MD-APCs according to any one of claims 1 to 3, which present adherent properties such as determined by the following test :

the macrophages are cultured for 2 h in culture medium (I.M.D.M. or R.P.M.I.) on plastic flasks and the percentage (%) of adherent cells is quantified for instance by microscopic analysis.

5. MD-APCs culture wherein :

- about 10% to about 50% of the MD-APCs present antigen CD14 on their surface,
 - about 10% to about 50% of the MD-APCs present antigen CD64 on their surface,
 - about 30% to about 100% of the MD-APCs present antigens CD80 and CD86 on their surface,
 - about 80% to about 100% of the MD-APCs present antigen MHC-II on their surface,
 - about 70% to about 100% of the MD-APCs present adherent properties,
 - about 30% to about 100% of the MD-APCs present a phagocytosis property,
- each MD-APCs having the above-mentioned properties being such that said properties are expressed according to the intensities as specified in any one of claims 1 to 4.

6. Process for preparing a composition comprising MD-APCs according to any one of claims 1 to 5, comprising the culture of mononuclear cells in a culture medium containing chemical ligands of mononuclear cells, such as histamine or histamine agonist and a H₂ antagonist, in combination or not with "additional" GM-CSF, or other chemical ligands interacting with mononuclear cells and allowing differentiation into MD-APCs, such as detoxified LPS such as lipid A, C3 and other ligands of complement receptors, taxols, oxydoreductors such as flavenoids or polyphenols, ligands to CD40, to the TNF receptors or to vitamin D3 receptors.

7. Process according to claim 6, wherein the culture medium contains chemical ligands of mononuclear cells, for example histamine and cimetidine or a H₂ antagonist without "additional" GM-CSF, histamine being present at a concentration of about 10⁻² M to about 10⁻⁶ M, preferably of about 10⁻⁴ M, and cimetidine or the H₂ antagonist being present at a concentration of about 10⁻⁴ M to about 10⁻⁹ M, preferably of about 10⁻⁶ M.

AMENDED SHEET

8. Process according to claim 6, wherein the culture medium contains chemicals ligands of mononuclear cells, for example histamine and cimetidine or a H₂ antagonist, in combination with "additional" GM-CSF, histamine being present at a concentration of about 10⁻² M to about 10⁻⁶ M, preferably of about 10⁻⁴ M, cimetidine or the H₂ antagonist being present at a concentration of about 10⁻⁴ M to about 10⁻⁹ M, preferably of about 10⁻⁶ M, and additional GM-CSF being present at a concentration of about 50 U/ml to about 2000 U/ml, preferably of about 500 U/ml.

9. Process according to any one of claims 6 to 8, comprising :

- isolation of leukocytes, from healthy donors or from patients, from peripheral blood by apheresis and removal platelets and anticoagulant from the apheresis product,
- isolation of mononuclear cells (monocytes + lymphocytes) from red cells and granulocytes in order to have less than 10% granulocytes and less than 5% red cells,
- culture of the mononuclear cells obtained at the previous stage by placing them in an appropriate culture medium containing chemical ligands of mononuclear cells, such as histamine or an agonist of histamine, an H₂ antagonist, such as cimetidine, in combination or not with GM-CSF, for a time sufficient to obtain differentiated MD-APCs, preferably for about 5 to 15 days, and possibly separating the MD-APCs from the lymphocytes, and recovering the MD-APCs or the macrophages and lymphocytes.

10. Process according to any one of claims 6 to 9, wherein the culture medium of MD-APCs is added

- with crude antigens, for instance autologous tumor membrane, killed tumoral cells, bacterial capsides, viral homogenates cleared from nucleic acids,
- specific peptides against which an immune response is desired,
- cDNA or genetic material linked to vectors (for example gluconated polylysine) to allow transfection of the MD-APCs with material coding for the relevant peptide or protein to be presented on the macrophage membrane and against which an immune response is desired,
- or bispecific antibodies targeting on the one side, a surface antigen of the MD-APCs and, on the other side, a relevant antigen against which an immune response is desired.

AMENDED SHEET

11. MD-APCs liable to be obtained according to the process of any one of claims 6 to 10.

12. Pharmaceutical compositions containing as active substance, MD-APCs according to any one of claims 1 to 5 or 11.

13. Cellular vaccine compositions containing as active substance, MD-APCs according to any one of claims 1 to 5 or 11.

14. Medium containing elements necessary for the growth and differentiation of monocytes into MD-APCs according to claims 1 to 5 or 11, and in addition containing chemical ligands of mononuclear cells, for example histamine, cimetidine in combination or not with GM-CSF.

15. Cell processor or kit containing :

- means for the recovery of lymphocytes and monocytes free of contaminants,
- appropriate buffer and wash solutions and possibly appropriate means for the conservation of MD-APCs,
- means for preparing a culture for the monocytes and possibly the lymphocytes and containing chemical ligands of mononuclear cells, for example histamine, cimetidine or a H₂ antagonist in combination or not with GM-CSF,
- possibly means for transfection of cultured cells and means for targeting antigens to MD-APCs.

16. Cell processor or kit according to claim 15, containing :

- means for recovering and centrifuging blood to obtain a leukocyte concentrate,
- means for separating lymphocytes and monocytes from the other white cells and for eliminating the contaminating red cells,
- culture medium for MD-APCs and possibly lymphocytes with complements and particularly chemical ligands of mononuclear cells, for example histamine and cimetidine or a H₂ antagonist, in combination or not with GM-CSF,
- appropriate means for the conservation of MD-APCs,
- appropriate buffer and wash solution.

17. Products containing MD-APCs according to any one of claims 1 to 5 or 11, and lymphocytes, as a combined preparation for simultaneous, separate or sequential use in cell therapy.

AMENDED SHEET

18. Products according to claim 17, characterized in that they contain the MD-APCs and the lymphocytes in a ratio of at least 20% to 50% of MD-APCs expressed in cell number.

5 19. Method for the clinical treatment, comprising the administration of an appropriate amount of MD-APCs according to any one of claims 1 to 5 or 11, and preferably in an amount of about 10^8 to about 5×10^9 MD-APCs.

10 20. Method according to claim 19 for the treatment of any disorder, comprising the administration of lymphocytes in an amount of about 4×10^9 to about 10×10^9 lymphocytes.

15 21. Use of chemical ligands of mononuclear cells, for example an agonist of histamine, in particular histamine, and a H_2 antagonist, in particular cimetidine, in combination or not with GM-CSF, for the preparation of MD-APCs having the following properties :

- they present on their surface :

* antigen CD14 and CD64 with a mean intensity of about 5 to about 200,

20 * antigen CD80 and CD86 with a mean intensity of about 20 to about 200,

* antigen CD40 and mannose receptor with a mean intensity of 50 to 500.

- they are substantially devoid of the surface antigens CD1a and CD1c, the presence and mean intensities respectively of CD14, CD64, CD80, CD86 and the absence of CD1a and CD1c being for instance determined by immunofluorescence staining and flow cytometry analysis,

25 - they present high phagocytosis property such as determined by the following test : said phagocytosis capacity being evaluated by an uptake of formalin fixed yeast, for example by culturing MD-APCs for 2 hours to select adherent cells, adding yeast in 1/10 mMD-APCs/yeast ratio and incubating at 37°C , 5% CO_2 atmosphere for 2-3 hours fixing by the May-Grünwald-Giemsa (MGG) staining, and the percentage of phagocytic MD-APCs being quantified for instance by microscopic analysis,

30 - they have the property of stimulating the proliferation of allogenic lymphocytes such as determined by the following test :

35 AMENDED SHEET

allogenic primary mixed lymphocytes reaction (MLR) was carried out in 96-well microtiter plates by adding different numbers (2×10^3 to 2×10^5 in 100 μ l medium/well) of MD-APCs to 2×10^5 in 100 μ l medium/well of allogenic T cells purified from buffy coats and after 5 days incubation at 37°C, cell proliferation was assessed by a colorimetric method, such as the cleavage of tetrazolium salt WST-1 (slightly red) to Formozan (dark red) or such as Brdu incorporation during DNA synthesis.

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AMENDED SHEET

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COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**NEW ANTIGEN PRESENTING CELL, A PROCESS FOR PREPARING THE SAME AND
THEIR USE AS CELLULAR VACCINES**

the specification of which: *(check one)*

REGULAR OR DESIGN APPLICATION

- ☐ is attached hereto.
- ☐ was filed on _____ as application Serial No. _____ and was amended on _____ (if applicable).

PCT FILED APPLICATION ENTERING NATIONAL STAGE

- ☒ was described and claimed in International application No. PCT/EP97/02703 filed on 15 May 1997 and as amended on _____ (if any).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

PRIORITY CLAIM

I hereby claim foreign priority benefits under 35 USC 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

PRIOR FOREIGN APPLICATION(S)

Country	Application Number	Date of Filing (day, month, year)	Priority Claimed
Europe	96401099.5	21 May 1996	Yes

(Complete this part only if this is a continuing application.)

I hereby claim the benefit under 35 USC 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 USC 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)

(Filing Date)

(Status—patented, pending, abandoned)

POWER OF ATTORNEY

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from Grosset-Fournier & Demachy as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

As a named inventor, I hereby appoint the following attorney(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: **Robert J. PATCH, Reg. No. 17,355, Andrew J. PATCH, Reg. No. 32,925, Robert F. HARGEST, Reg. No. 25,590, Benoît CASTEL, Reg. No. 35,041, Eric JENSEN, Reg. No. 37,855, Thomas W. PERKINS, Reg. No. 33,027, and Roland E. LONG, Jr., Reg. No. 41,949, c/o YOUNG & THOMPSON, Second Floor, 745 South 23rd Street, Arlington, Virginia 22202.**

Address all telephone calls to Young & Thompson at 703/521-2297.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor: **Mohamed CHOKRI**
(given name, family name)

Inventor's signature M. Chokri

Date November 10, 1998

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Date 12 November 1998

Residence: **Bures-Sur-Yvette, France**

Citizenship: **French**

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Full name of third joint inventor, if any: **Jean-Loup ROMET-LEMONNE**
(given name, family name)

Inventor's signature J. Romet-Lemonne

Date 12 Nov 1998

Residence: **Paris, France**

Citizenship: **French**

Post Office Address: **8, rue de Hesse
F-75003 Paris, France**

Form B

VERIFIED STATEMENT CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) & 1.27(c))--SMALL BUSINESS CONCERN

 Docker Number (Optional)
 USB 96 AK IDM APC
Applicant or Owner: Mohamed CHOKRI et al.

Serial or Patent No.: _____

Filed or Issued: _____

 Title: NEW ANTIGEN PRESENTING CELL, A PROCESS FOR PREPARING
THE SAME AND THEIR USE AS CELLULAR VACCINES

I hereby declare that I am:

- ☐ the owner of the small business concern identified below;
- ☐ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF SMALL BUSINESS CONCERN I.D.M. IMMUNO-DESIGNED MOLECULESADDRESS OF SMALL BUSINESS CONCERN 172, rue de CharonneP-75011 Paris, France

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 37 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention described in:

- ☐ the specification filed herewith with title as listed above.
- ☒ the application identified above.
- ☐ the patent identified above.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights in the invention must file separate verified statements averring to their status as small entities, and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization having any rights in the invention is listed below:

- ☐ no such person, concern, or organization exists.
- ☐ each such person, concern or organization is listed below.

Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING ROMET-LEMONNE Jean-LoupTITLE OF PERSON IF OTHER THAN OWNER V.P. Directeur GénéralADDRESS OF PERSON SIGNING 172 Rue de Charonne PARIS 75011SIGNATURE [Signature] DATE Nov. 12 1998